

Genetic Linkage Map Construction in Pearl Millet Using SSR Markers

Chelpuri Durgaraju¹, Rakesh K Srivastava³, Narayan Reddy², Pooja Katiyar³ and Kanaka Durga Kilaru^{2*}

¹College of Agriculture, ANGRAU, Rajendranagar, Hyderabad, 500 030

²Seed Research and Technology Centre, PJTSAU, Rajendranagar, Hyderabad, 500 030

³International Crops Research Institute for the Semi-Arid Tropics, Patancheru, 502324

⁴Department of Plant Pathology, College of Agriculture, Rajendranagar, 500 030

*Corresponding Author E-mail: kanakakilaru@yahoo.com

Received: 7.07.2017 | Revised: 1.08.2017 | Accepted: 5.08.2017

ABSTRACT

For pearl millet production in the semi-arid tropics, *Sclerospora graminicola* is a major biotic constraint causes Downy mildew. The pathogen is heterothallic and frequent recombination leads to evolution of new virulent populations. Identification of resistance to new virulent isolates is a prerequisite for resistance breeding. A very good understanding of ability to manipulate oligogenic and quantitative traits is offered to plant breeders by recent advances in genetic marker technology. In the present investigation genotypic data generated for a total of 88 marker loci (39 Genomic SSRs and 49 EST SSRs) were used to construct a linkage map of the pearl millet mapping population of 188 F₈ RIL progenies based on the cross ICMB 89111-P6 x ICMB 90111-P6. A skeleton linkage map of seven linkage groups with a total map length of 725.5 cM (Haldane units) was constructed using data from 74 marker loci for 188 RILs using JoinMap at LOD threshold value of 5.0 and MapMaker/Exp version 3.0b and map was drawn using Map Chart 2.2. The map length of individual linkage groups ranged from a minimum of 32.1cM (LG3) to a maximum of 140.2 cM (LG1). The average inter marker distance was 9.8 cM, with an average density of 0.102 markers/cM. The total number of mapped loci per linkage group (LG) ranged from 5 on LG3 to 23 on LG1.

Key words: Linkage Map; Polymerase Chain reaction; mapping population.

Abbreviations: LG – Linkage group; PCR – Polymerase Chain reaction; SSR – Simple sequence repeats.

INTRODUCTION

Pearl millet is important as a staple food grain and source of feed and fodder for livestock, in hot, dry marginal agricultural production environments of Africa and South Asia that

are home to hundreds of millions of the world's poorest farmers. Downy mildew is the most destructive disease in India, caused by pathogen *Sclerospora graminicola* [(Sacc). Schroet] in pearl millet.

Cite this article: Durgaraju, C., Srivastava, R.K., Reddy, N., Katiyar, P. and Kilaru, K.D., Genetic Linkage Map Construction in Pearl Millet Using SSR Markers, *Int. J. Pure App. Biosci.* 5(5): 1304-1315 (2017). doi: <http://dx.doi.org/10.18782/2320-7051.5663>

The important diseases that are responsible for economic losses in pearl millet are downy mildew, blast, smut, ergot and rust. Downy mildew is the most destructive disease in India, which is also referred to as green ear disease, where the grains are replaced by leaf-like structures in the ear head. The estimated annual grain yield loss due to DM is approximately 20–40%²³ but this could be much higher under favorable conditions of disease development.

Pearl millet is a good experimental plant for genetic studies because of its low diploid chromosome number ($2n=14$) with a moderately high DNA content of $IC = 2.36$ pg. Its short duration (60-90 days), high genetic variability and its protogynous nature which facilitates control pollination are advantageous features. It has also been recognized as a well suitable crop for molecular studies.

The pearl millet downy mildew pathogen *Sclerospora graminicola* is an obligate biotrophic oomycete which reproduces asexually by means of sporangia that germinate to release motile zoospores and sexually through soil-borne oospores. It is known to be a highly variable pathogen. Stability of resistance to downy mildew in pearl millet inbred lines and hybrid cultivars has been elusive in India due to host-directed evolution of pathogenic variation in *S. graminicola* populations²². There are currently several pathogenic variants of *S. graminicola* prevalent in different parts of pearl millet growing regions of India and new variants with higher virulence levels emerge with deployment of new cultivars.

As the host is a crop of poor and marginal areas, the use of resistant cultivars is the most appropriate control method for pearl millet downy mildew in an integrated disease management system. Breeding for resistance to diseases contributes to increased productivity and stability of pearl millet grain, stover and forage yields. Hence, resistance breeding is a major component in pearl millet improvement programme.

A very good understanding of and ability to manipulate oligogenic and

quantitative traits is offered to plant breeders by recent advances in genetic marker technology²⁹. Molecular biology can be used to guide traditional plant breeding. Now hundreds of molecular markers have been created and detailed marker based genetic maps were produced for which the traits could be linked.

The first molecular marker-based genetic linkage map of pearl millet was built with restriction fragment length polymorphisms (RFLPs), the marker system of choice in the early 1990s. This map has served as the base for subsequent pearl millet marker-based studies at the John Innes Centre (JIC)⁸. Microsatellites remain the markers of choice for practical breeding applications with several advantages over RFLP, RAPD and AFLP markers. In addition, microsatellites exhibit co-dominant inheritance and their detection are readily automated. SSR markers are one of the best options available for foreground selection in marker-assisted backcrossing (MABC) programmes because they are hyper-variable, highly reproducible and readily multiplexed. They are also ideal for anchoring molecular linkage maps¹⁸.

The building up of a saturated molecular map using molecular markers like restriction fragment length polymorphisms (RFLPs) and microsatellites (SSR) makes it possible to dissect Mendelian factors underlying a complex trait such as disease resistance and consequently enhance the effectiveness and accelerate the rate of breeding programmes to improve pure line varieties of self-pollinated crops and hybrid parental lines of cross-pollinated crops. Linkage drag and confounding effects of environmental variation associated with conventional breeding can also be reduced¹⁴. Therefore, in addition to breeding for yield contributing characters, more attention should be given for breeding resistant varieties and hybrids with higher yield. Disease resistance in high yielding varieties ensures farmers less dependence on chemical control and also avoids environmental pollution.

MATERIAL AND METHODS

Mapping population

RIL mapping population of F_8 generation with size of 188 genotypes from the cross ICMB 89111-P6 \times ICMP 423-P6 (ICMB 90111-P6) was directly provided for the work by Pearl Millet Molecular Breeding Section, Dry Land Cereals, ICRISAT, Patancheru.

DNA extraction

Several procedures for genomic DNA isolation have been reported⁷; Murray and Thompson¹⁴ and Tai and Tanksly²². The procedure based on CTAB extraction buffer¹³ was used for pearl millet genomic DNA isolation in this study.

The seedlings of parents and mapping population progenies were grown in a growth room in 6 cm diameter hydrated Jiffy-7 pellets (P/N 31130100, Jiffy International, Norway) placed in plastic trays and watered for every 2 days. Around 30 selfed seeds from each of 188 F_8 progenies and both parents were grown in pots under dark conditions. Bulk DNA was obtained from approximately 30 mg representative sample per F_8 progeny and parental lines by using CTAB method¹³ with slight modifications. DNA was further purified by RNase digestion followed by extraction with phenol: chloroform: Iso-amyl alcohol (25:24:1) and ethanol precipitation as described¹³.

Parental Polymorphism and Genotyping the RIL Population

According to Caetano-Anolles⁵, the parameters of DNA amplification (*viz.*, specificity, efficiency and fidelity) are strongly influenced by the different components of the reaction and by thermal cycling. Therefore careful optimization of these parameters will ultimately result in reproducible and efficient amplification.

PCR conditions for pearl millet SSR primers were optimized using a grid with various amounts and concentrations of chemicals used for the PCR master mix, at different annealing temperatures. Among the SSR markers used, the PSMP series genomic SSR markers were obtained from Qi *et al*^{15,16} and Allouis *et al*², CTM series markers

were obtained from Budak *et al*⁴, ICMP series EST-SSR markers were published¹⁹ and IPES series EST-SSR markers were published¹⁸. To identify SSR primer pairs detecting polymorphism between parents, initial screening of parental lines was conducted. For this, DNA was extracted from ICMB 89111-P6 (taken as first parent *i.e.* P1) and ICMB 90111-P6 (taken as second parent *i.e.* P2). A total of 468 SSR primers were used to screen the parents. From this screening, 88 SSR primers detecting scorable polymorphism between the parents were noted and used for genotyping of the RIL mapping population of these parents.

Polymerase Chain Reaction (PCR)

PCR reactions were conducted in 96 and 384-well plates in a GeneAmp PCR system PE 9700 (Applied Biosystem, USA) DNA thermal cycler in volumes of 5 μ l. A touchdown PCR program was used to amplify the DNA fragments. Reaction conditions are as follows. Initial denaturation for 5 minutes at 94°C (to minimize primer - dimer formation and to activate the *Taq* polymerase), subsequently 10 cycles of denaturation for 15 seconds at 94°C, annealing at 61°C to 52°C for 20 seconds, the annealing temperature for each cycle is reduced by 1°C and extension at 72°C for 30 seconds followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds followed by final extension at 72°C for 20 min. PCR amplification was checked on 1.2% agarose gels and PCR products of direct labelled primers and M_{13} tailed primers were separated by capillary electrophoresis on an ABI3730xl sequencer and their sizes were determined using GeneMapper[®] Version 4.0 software (Applied Biosystems, USA) and PCR products of unlabelled primers were separated on PAGE and Agarose.

Polyacrylamide Gel Electrophoresis

The PCR products of SSR and EST-SSR markers were separated on 6% non-denaturing PAGE. The materials required were sequencing gel apparatus (glass plates, spacers, casting apparatus) and combs (101 wells). The reagents required are detailed in

Appendix 2 and the protocol used for PAGE gel preparations is as follows.

A. For 6% gel (plate size 38 × 30 cm) 75 ml of gel solution would be sufficient.

- a. 10X TBE buffer 8 ml.
- b. V/V Acrylamide/ Bisacrylamide (29:1) 15.0 ml.
- c. Distilled water 52 ml.

The above ingredients are mixed in 200 ml Erlenmayer flask.

B. The above solution mixed vigorously and 450 µl APS was added with swirling the flask and then immediately 100 µl TEMED was added and mixed. Then the solution was poured using a syringe (100 ml) that fits into the slot between the glass plates and comb was inserted (upside down, in order to form wells). The gel matrix was allowed to polymerize for 30 minutes. Gels can be stored overnight as long as the plate ends are wrapped in pre-wetted tissue paper (1X TBE) and covered with plastic film.

C. After polymerization, comb was removed from gel and kept for running. The lower tank and reservoir were filled with 0.5X TBE buffer (approximately 250-300 ml). Ensure that wells are covered with the buffer. The wells were cleaned by aspirating and dispensing 0.5X TBE buffer on the wells using a Pasteur pipette to remove small fragments of gel and tiny bubbles from the top of the wells. The comb was inserted back on top of the wells (comb tips should rest on the well (<1 mm deep), don't force it into the well).

D. The gel was put for pre-run to warm it, for at least 10 minutes at 5 V/cm (400 V, 9 W, depending on width of the gel).

E. The samples were made ready for loading with 5X loading buffer to give a final concentration of 1X (for instance 2 µl sample + 2 µl water + 1 µl 5X loading buffer).

F. The gel was put for run at approximately 5 V/cm (400 V, 9 W, depending on width of the gel). Higher voltage causes the gel to overheat and result in un-even run of the samples. The gel was run until the desired resolution has been reached (front of the tracking dye should reach a few cm above the end of the gel)

G. After the run, the plates were carefully pulled apart, so that the gel is attached to the front plate and start silver staining.

After running the PAGE gel electrophoresed DNA fragments were visualized using a modified silver staining procedure⁹. Several protocols for silver staining can be used, most of which require approximately 2 h. although commercial kits for silver staining were available from several manufacturers (e.g., Bio-Rad), lab-made solutions were used in the present study. Each solution was prepared in a separate container. The same solutions were used twice over a 30 h period except for silver nitrate solution and developer, which were freshly prepared during the staining process. The following steps were adopted for silver staining:

1. Rinse the gel in 2 liters of distilled water for 5 minutes.
2. Soak the gel in 2 liters of 0.1% CTAB for 20 minutes (2 g in 2 litre of water) with shaking.
3. Incubate the gel in 2 liters of 0.3% ammonia for 15 minutes (26 ml in 2 litre) with shaking.
4. Silver nitrate solution was prepared (2 g silver nitrate, 8 ml of 1M NaOH, 8 ml 25% ammonia) and titrated with ammonia until the solution became clear after which a further 1 ml of ammonia solution was added.
5. The gel was placed in the silver nitrate solution for 15 minutes and was gently agitated.
6. The gel was then rinsed in water for 1 minute.
7. The gel was placed in developer (30 g sodium carbonate, 0.4 ml formaldehyde, 2 litre of water) until the bands became visible.
8. The plate was rinsed in water for 1 minute to stop staining.

After silver staining of the gel, it was placed on a bench-viewer. The size (in base pairs) of the parental alleles for each SSR were estimated based on their migration relative to the 100 bp DNA ladder (fragments ranging from 100 bp to 1000 bp), and presence of parental alleles in each of the F₈ progenies were scored. The dried gel was removed (after scoring) from the front glass plate by soaking

in concentrated NaOH solution for a few hours.

Genotyping using capillary electrophoresis

The PCR products amplified using fluorescence-labeled primers genomic SSRs were separated by capillary electrophoresis using an ABI Prism 3700 automatic DNA sequencer (Applied Biosystems Inc.). This has the ability to detect size differences of 1 bp using a fluorescence-based detection system, thus dispensing with the need for radioactivity or laborious manual Polyacrylamide gel techniques.

For this purpose, forward primers were labelled with 6-FAM™ (Blue), VIC™ (Green), NED™ (Yellow) or PET™ (Red) fluorophores (Applied Biosystems). PCR products of primers labelled with different dyes or same fluorophore-labelled primers with non-overlapping amplicons (in terms of size) were pooled (post-PCR). The products of different fluorophore-labelled primers were pooled in different proportion (1.0 μ l of 6-FAM-labelled product, 0.8 μ l of VIC-labelled product, 1.4 μ l of NED-labelled product, and 1.0 μ l of PET-labelled product). The pooled PCR products were then mixed with 0.2 μ l of GeneScan 500™ LIZ® internal size standard (Applied Biosystems) and 7.0 μ l of Hi-Di™ Formamide (Applied Biosystems). The final volume was made up to 15 μ l with sterile double-distilled water. DNA fragments were denatured for 5 minutes at 95°C (Perkin Elmer 9700, Applied Biosystem) and cooled immediately on ice.

Fragment size fractionation

The PCR products with denatured DNA were electrophoresed and the capillary run was performed using the “Genscan2 POP6 Default” run module and “G5” filter-set. The analysis module used was “GS500 analysis”. The fragments were separated in a 50 cm capillary array using POP6 (Performance Optimized Polymer, Applied Biosystems) as separation matrix.

Data Processing

GeneMapper® version 4.0 software (Applied Biosystems, USA) was used to size the peak pattern in relation to the internal size standard,

GeneScan 500™ LIZ®. The principle behind this is that standards are run in the same lane or capillary injection as the samples, which contain fragment of unknown sizes labeled with different fluorophores. GeneMapper® version 4.0 Software automatically calculates the size of unknown DNA fragments by generating a calibration sizing curve based upon the migration times of the known fragments in the standard. The unknown fragments are mapped on to the curve and the sample data is converted from migration times to fragments size. The peaks were displayed with base pair values and height (amplitude) in a chromatogram. The height of the chromatogram peaks (representing the alleles) obtained through capillary electrophoresis is directionally proportionate to the signal strength, which in turn is determined by the amount of amplified product in the sample.

Scoring of amplified bands

The banding pattern of each of amplified PCR products of various marker systems were scored as follows:

A = Homozygote for the allele for downy mildew susceptible parent at a locus.

B = Homozygote for the allele for downy mildew resistant parent at a locus.

H = Heterozygote carrying the alleles from both parents.

- = Missing data for the individual at a locus.

After scoring individual progenies, the data set was assembled in Microsoft Excel spreadsheet in a format suitable for linkage analysis by JoinMap and Mapmaker (*i.e.* rows = genotype score at a given locus; columns = individual F₈ progenies).

Linkage map construction

Marker classes at each locus were summarized for all individuals into two different genotypic classes expected for an F₈ (RIL) population. The segregation of each marker was tested with a chi-square test for goodness of fit to the expected Mendelian segregation ratio (1:1) of the parental configuration. The linkage map was constructed with JoinMap²¹ and MAPMAKER/Exp version 3.0b supplied by E.S. Lander, Whitehead Institute for Biomedical Research, Cambridge,

Massachusetts, USA¹². The Haldane¹¹ mapping function was used to convert the recombination frequencies to genetic distances in centiMorgans (cM). The consensus maps of pearl millet constructed by Qi *et al*¹⁶ and Rajaram *et al*¹⁸ were used for comparison. The analysis was carried out by evaluating the mapping population as an F₈ population using two point analysis to identify linked pairs at a LOD score of 5.0. In Mapmaker, the 'Sequence all' command was used for two-point (or pair-wise) linkage analysis, while the 'Group' command was used to group the marker in a sequence into different linkage groups. The 'Compare' command was used to calculate the maximum likelihood map for each specified order of markers and reported the orders sorted by likelihood of their maps. One sequence can specify more than one order of loci. The order having a log-likelihood of 0.0 was selected as the best order. The obtained order was then analyzed further using a three-point linkage analysis via the 'Ripple' command. Other markers were added using the 'Try' command and their positions were fine-tuned using the 'Ripple' command. The 'Ripple' command was used to assign exact

orders of markers. Majority markers used in the present study have been already mapped in previous studies and they were used as reference for the map obtained in the present study to compare the linkage distance, marker position and marker order in linkage groups. Markers used in the present study were therefore assigned to linkage groups based on their known chromosome locations and their order in the present F₈ mapping population was verified using MAPMAKER. After getting the position and distance between markers linkage map was drawn using Map Chart 2.1²⁵.

RESULTS AND DISCUSSION

Genotypic data generated for a total of 88 marker loci (39 Genomic SSRs and 49 EST SSRs) were used to construct a linkage map of the pearl millet mapping population of 188 F₈ RIL progenies based on the cross ICMB 89111-P6 x ICMB 90111-P6. A previously constructed integrated consensus pearl millet linkage map¹⁸ based on SSR and EST-SSR markers was used as a reference for marker linkage group assignment and initial marker ordering in this study.

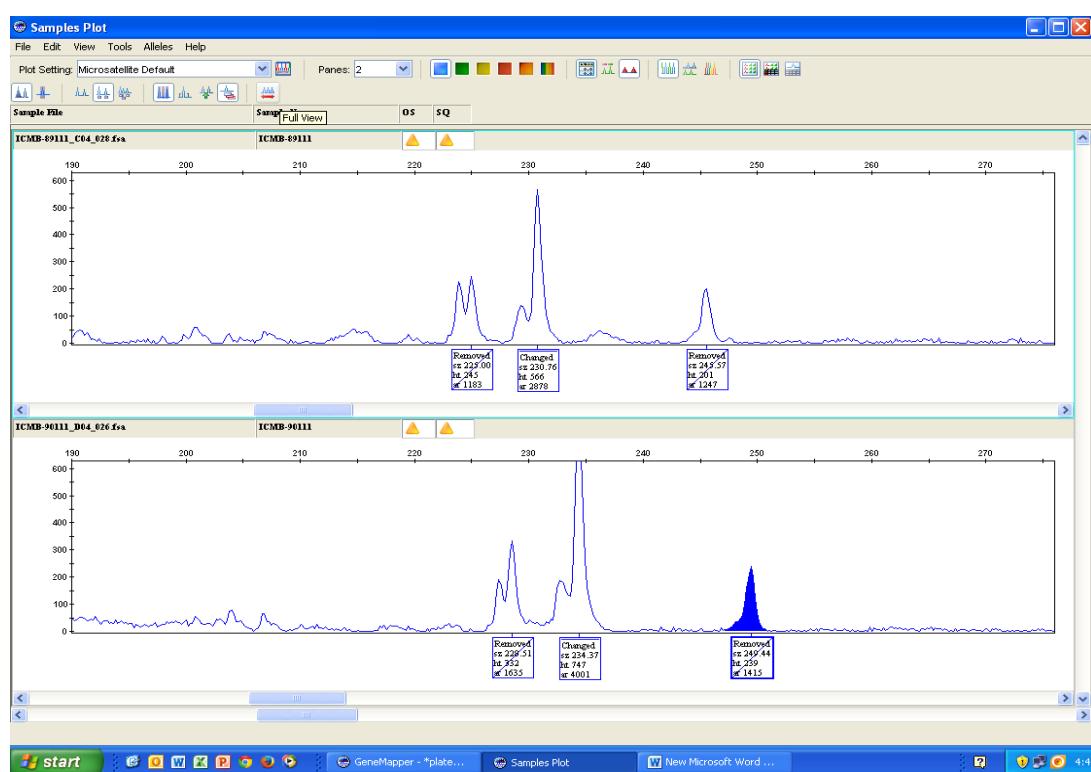


Fig. 1: Gene Mapper profile for an amplified SSR marker showing polymorphism

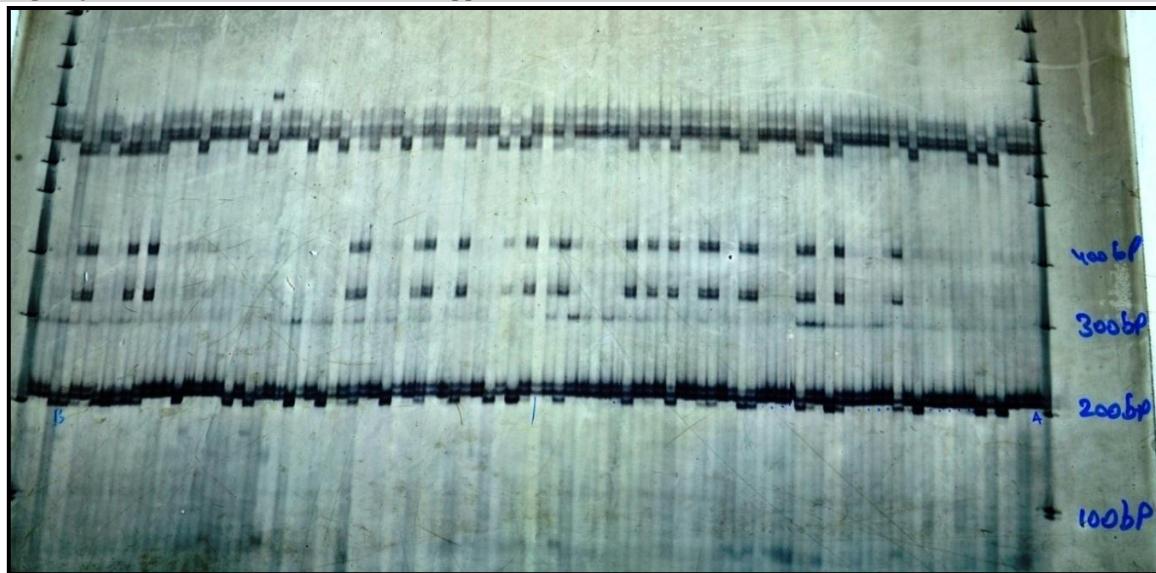


Fig. 2: PAGE Gel image showing polymorphism for Xipes0195 primer

A linkage map of seven linkage groups with a total map length of 725.2 cM was constructed using data from 74 marker loci for 188 F₈ progenies. The map lengths of individual linkage groups ranged from a minimum of 32.1cM (LG3) to a maximum of 140.2 cM (LG1), as shown in figure.3. The average inter marker distance was 9.8 cM, with an average density of 0.102 markers/cM. The total number of mapped loci per linkage group (LG) ranged from 5 on LG3 to 23 on LG1.

JoinMap version 4.1²¹ was used to construct the linkage map using a LOD

threshold value of 5.0 and recombination fraction of 0.5. After making seven linkage groups, markers which are coming in sub groups and markers which are unlinked were then placed in appropriate linkage groups using the 'build' and 'try' command of MAPMAKER/EXP version 3.0b. Markers with satisfactory orders were then anchored in each linkage group and the frame work command was used. Marker orders with fewer candidate errors and higher log likelihood (LOD) values were preferred for anchoring and frame working of each linkage group.

Table 1: Linkage group details of genomic SSR and EST-SSR based genetic map for pearl millet RIL population based on cross ICMB 89111-P6 x ICMB 90111-P6.

Linkage group	Total number of markers	SSRs		MAP LENGTH (cM)
		Genomic	EST	
LG1	23	6	17	140.2
LG2	12	9	3	124.8
LG3	5	3	2	32.1
LG4	6	3	3	129.7
LG5	12	8	4	122.0
LG6	6	3	3	101.2
LG7	10	5	5	75.2
TOTAL	74	37	37	725.2

Linkage Group1

The total length of Linkage group1 is 140.2 cM, which is longest among the seven linkage groups. It accommodated 23 loci detected by 6 Genomic SSRs (Xpsmp2273, Xpsmp2030, Xctm57, Xpsmp2080, Xpsmp2218 and Xpsmp2232) and 17 EST-SSRs (Xipes0098, Xipes0203, Xipes0126, Xipes0146, Xicmp3017, Xipes0045, Xipes0101, Xipes0227, Xipes0004, Xicmp4010, Xipes0221, Xipes0214, Xicmp3088, Xipes0061, Xipes0124, Xipes0127 and Xipes0162). The total map length of this linkage group is 140.2 cM. The "compare" command of MapMaker predicted the best marker order of Xpsmp2273, Xpsmp2030, Xpsmp2080, Xpsmp2218, Xipes0203, Xipes0126, Xipes0146, Xicmp3017, Xipes0045, Xipes0101, Xipes0004, Xicmp3088 and Xipes0127 with inter-marker map distances ranging from 0.9 cM between Xipes0098 and Xpsmp2273 to 19.2 cM between Xipes0127 and Xipes0162 (fig. 3).

The length of LG1 was comparable with the reference map's LG1 (146.6cM). In this map out of 23 markers, 16 markers were mapped to LG1 in consensus map. Markers which are unmapped in consensus map (Xctm57, Xipes0124, Xipes0227 and Xipes0061) and markers which mapped to different linkage groups (Xipes0162 and Xipes0221 on LG2 and Xipes0214 on LG5) are mapped to LG1 in this present study, because they are showing strong linkage to LG1 markers up to LOD 10 in JoinMap v4.1.

Linkage Group2

The total length of linkage group2 is 124.8 cm. It consists of 9 Genomic SSRs (Xpsmp2237, Xpsmp2072, Xpsmp2088, Xpsmp2077, Xpsmp2201, Xpsmp2206, Xctm21, Xpsmp2231 and Xpsmp2089) and 3 EST-SSRs (Xipes0181, Xipes0007 and Xipes0117). The "compare" command of MapMaker predicted the best marker order of Xipes0007, Xpsmp2088, Xipes0117, Xpsmp2206 and Xctm21 with inter-marker map distances ranging from 0.1 cM between Xpsmp2072 and Xpsmp2088 to 53.6 cM between Xpsmp2231 and Xpsmp2089 (fig.3).

In this map all the 12 markers were mapped to LG2 in consensus map. The length of LG2 was smaller than the reference map's LG2 (192.6cM) may be because of less number of markers and lack of markers in distal region from the top of the consensus map¹⁸.

Linkage Group3

This is the shortest linkage group among the seven in this skeleton map. It has a total length of 32.1 cM and consists of 3 Genomic SSRs (Xctm10, Xpsmp2070 and Xpsmp2267) and 2 EST-SSRs (Xipes0223 and Xipes0180). The "compare" command of MapMaker predicted the best marker order of Xipes0180 with inter-marker map distances ranged from 3.4 cM between Xpsmp2070 and Xpsmp2267 to 13.4 cM between Xipes0223 and Xpsmp2070 (fig.3).

In this map all the 5 markers were mapped to LG3 in consensus map. The length of LG3 was much smaller than the reference map's LG3 (94.4 cM) may be because of less number of markers and lack of markers in distal region from the top of the consensus map¹⁸.

Linkage Group4

The total length of linkage group4 is 129.7 cM, which is the second longest among the seven linkage groups. It accommodated 6 loci detected by 3 genomic SSRs (Xpsmp2008, Xpsmp2084 and Xpsmp2081) and 3 EST-SSRs (Xipes0114, Xipes0174 and Xipes0208). The best order predicted by "compare" command of MapMaker is Xipes0208 with inter-marker map distances ranging from 7.3 cM between Xipes0114 and Xpsmp2084 to 40.4 cM between Xipes0208 and Xpsmp2081 (fig.3).

In this map all the 6 markers were mapped to LG4 in consensus map. The length of LG4 was larger than the reference map's LG4 (87.4 cM) may be because of large interval between the marker which is located in the centre (Xpsmp2081) to the marker which is located in the distal region (Xipes0208) and the larger intervals between the markers.

Linkage Group5

The total length of linkage group5 is 122 cM, which is next large to LG 4. It accommodated

12 loci detected by 8 Genomic SSRs (Xpsmp2078, Xpsmp2202, Xpsmp2274, Xpsmp2220, Xpsmp2261, Xpsmp2277, Xpsmp2276 and Xpsmp2208) and 4 EST SSRs (Xicmp3027, Xipes0230, Xipes0093 and Xicmp3085). The best order predicted by "compare" command of MapMaker is Xipes0230 and Xipes0093 with inter-marker map distances ranged from 2.5 cM between Xpsmp2276 and Xpsmp2277 to 22.5 cM between Xpsmp2220 and Xpsmp2261 (fig.3). In this map all the 12 markers were mapped to LG5 in consensus map. The length of LG5 was smaller than the reference map's LG5 (134.5 cM) may be because of less number of markers and lack of markers from distal region from top in the consensus map¹⁸.

Linkage Group6

The total length of linkage group6 is 101.2 cM. It accommodated 6 loci detected by 3 Genomic SSRs (Xpsmp2225, Xpsmp2248 and Xpsmp2213) and 3 EST SSRs (Xipes0167, Xipes0187 and Xipes0087) with inter-marker map distances ranged from 5.7 cM between Xpsmp2248 and Xpsmp2213 to 69.3 cM between Xipes0087 and Xpsmp2248 (fig.3). The length of LG6 was comparable with the reference map's LG6 (113.7 cM). In this map out of 6 markers, 4 markers were mapped to LG6 in consensus map. Marker which is unmapped in consensus map (Xipes0187) and marker which mapped to different linkage

group (Xpsmp2225 on LG2) are mapped to LG6 in this present study, because they are showing strong linkage to LG6 markers up to LOD 10 in JoinMap v4.1.

Linkage Group7

The total length of linkage group7 is 75.2cM, which is next small to LG 3. It accommodated 10 loci detected by 5 Genomic SSRs (Xpsmp2087, Xpsmp2203, Xpsmp2224, Xpsmp2229 and Xpsmp2263) and 5 EST SSRs (Xicmp3066, Xipes0105, Xipes0115, Xipes0195 and Xipes0097). The best order predicted by "compare" command of MapMaker is Xipes0105, Xpsmp2087, Xpsmp2224 and Xpsmp2229 with inter-marker map distances ranged from 2.2 cM between Xpsmp2224 and Xpsmp2229 to 13.6 cM between Xipes0097 and Xpsmp2087 (fig.3).

The length of LG7 was smaller than the reference map's LG7 (75.2 cM) may be because of less number of markers and lack of markers from distal region from top in the consensus map¹⁸. In this map out of 10 markers, 8 markers were mapped to LG7 in consensus map. Markers which are unmapped in consensus map (Xicmp3066 and Xipes0115) are mapped to LG7 in this present study, because they are showing strong linkage to LG7 markers up to LOD10 in JoinMap v4.1.

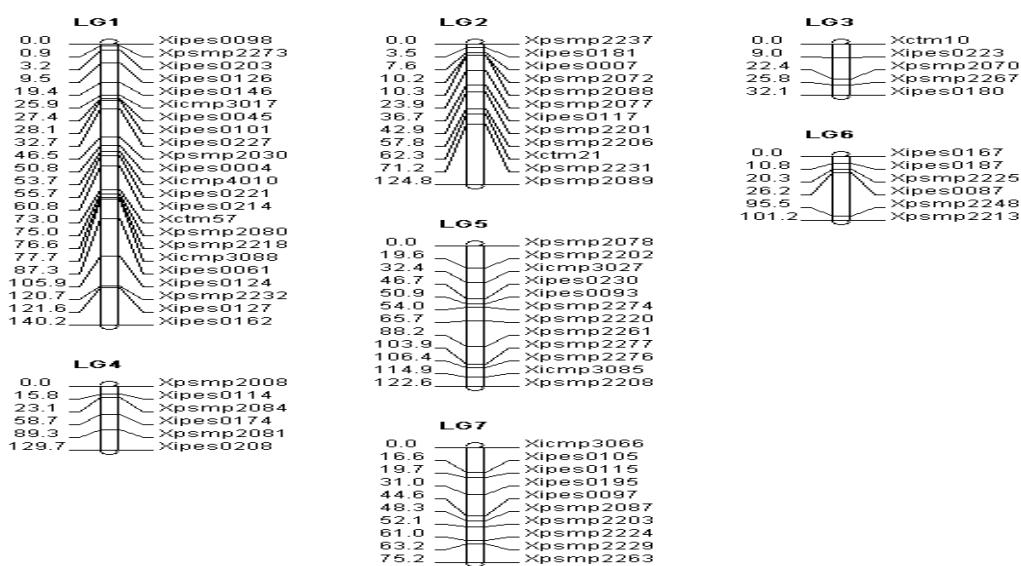


Fig. 3: Linkage map drawn from genotypic data of 188 F₈ RILs based on Cross ICMB 89111-P6 x ICMB 90111-P6

The construction of genetic linkage maps and QTL mapping for economical traits in fields crops are very important tools for studying genome structure, identifying introgression between genomes and localizing genes of interest in genomic regions.

The reference consensus map developed by Rajaram *et al*¹⁸, using four F₇ recombinant inbred populations (RIP) based on crosses ICMB 841-P3 × 863B-P2 (RIP A), H 77/833-2 × PRLT 2/89-33 (RIP B), 81B-P6 × ICMP 451-P8 (RIP C) and PT 732B-P2 × P1449-2-P1 (RIP D) is having length of 899 cM detected by 169 primer pairs. In this map majority of the markers that were mapped are mapped to same linkage group in the consensus maps of Rajaram *et al*¹⁸ and Qi *et al*¹⁶, except for swapping of some marker orders within some blocks on a few linkage groups. Such differences in marker order among genetic maps is not unexpected, as genetic mapping only gives an indication of the relative positions and genetic distances of the markers to each other²⁰. The relatively large population sizes (188 RILs from ICMB 89111-P6 x ICMB 90111-P6) used for construction of the genetic linkage map presented here as compared to studies where 62 and 120 RILs used in pearl millet by Quarrie *et al*¹⁷ and Akbari *et al*¹, respectively is highly advantageous for further exploitation of these maps. These larger population sizes improve the estimation of marker orders, which in turn improves the resolution of QTL mapping of polygenic traits. They also enable a greater resolution in the positioning of QTLs on the genetic map, while distribution of markers across the full length of the genome is required to detect all contributing loci⁶. However, it will be useful to use larger population sizes with such large numbers of markers in order to get more accuracy in marker order of these maps.

Once linkage between a marker locus and the gene for an agronomic trait of interest has been established, DNA diagnostic tests can be used to guide plant breeding as a substitute for other phenotypic tests such as assays for disease resistance. The development and availability

of abundant, naturally occurring molecular genetic markers (RFLP, RAPD and isozymes etc.,) during last two decades has generated renewed interest in counting, locating and measuring the effects of genes (polygenes or QTLs) controlling quantitative traits³. This linkage map can be used for the identification of Quantitative TraitLoci, for the resistance breeding programme in the Pearl Millet breeding study.

Acknowledgements

The first and second authors thank Acharya N.G. Ranga Agricultural University (ANGRAU) for financial help in the form of stipend during the course of work.

REFERENCES

1. Akbari, M., Wenzl, P., Caig, V., Carling, J., Xia, L., Yang, S., Uszynski, G., Mohler, A., Lehmensiek, A., Kuchel, H., Hayden, M.J., Howes, N., Sharp, P., Vaughan, P., Rathnayake, B., Huttner, E and Kilian, A. Diversity arrays technology (DArT) for high throughput profiling of the hexaploid wheat genome. *Theory of Application Genetics*. **113**: 1409-1420 (2006).
2. Allouis, S., Qi, X., Lindup, S., Gale, M.D and Devos, K.M.. Construction of a BAC library of pearl millet, *Pennisetum glaucum*. *Theory of Application Genetics*. **102**: 1200-1205 (2001).
3. Azhaguvvel and P. Linkage map construction and identification of QTLs for downy mildew (*Sclerospora graminicola*) resistance in pearl millet [*Pennisetum glaucum* (L.) R. (2001).
4. Budak, H., Pedraza, F., Cregan, P.B., Baenziger, P.S and Dweikat, I. Development and utilization of SSRs to estimate the degree of genetic relationships in a collection of pearl millet germplasm. *Crop Science*. **43**: 2284-2290 (2003).
5. Caetano-Anolles, G., Bassam, B.J. and Gresshoff, P., DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology*. **9**: 553-557.(1991)

6. Chalmers, K.J., Campbell, A.W., Kretschmer, J., Karakousis, A., Henschke, P.H., Pierens, S., Harker, N., Pallotta, M., Cornish, G.B., ShariXou, M.R., Rampling, L.R., McLauchlan, A., Daggard, G., Sharp, P.J., Holton, T.A., Sutherland, M.W., Appels, R and Langridge, P. Construction of three linkage maps in bread wheat, *Triticum aestivum*. *Australian Journal of Agricultural Research*. **52**: 1089-1119 (2001).
7. Dellaporta, S.L., Wood, J and Hicks, J.B. A plant DNA mini preparation. *Plant Molecular Biology Reporter*. **1**: 19-21 (1983).
8. Devos, K.M., Pittaway, T.S., Reynolds, A and Gale, M.D. Comparative mapping reveals a complex relationship between the pearl millet genome and those of foxtail millet and rice. *Theoretical and Applied Genetics*. **100**: 190-198 (2000).
9. Goldman, D and Merrill, C.R. Silver staining of DNA in polyacrylamide gels. *Electrophoresis*. **3**: 24-26 (1982).
10. Gulia, S.K. 2004. QTL mapping for improvement of downy mildew [*Sclerospora graminicola* (sacc.) J. schroet.] resistance (DMR) in pearl millet (*Pennisetum glaucum* (L.) R. Br.) hybrid parental line ICMB 89111. *Ph. D Thesis*. Chaudhary Charan Singh Haryana Agricultural University, Hisar, India.
11. Haldane, J.. The combination of linkage values and the calculation of distance between the loci of linked factors. *Journal of Genetics*. **8**: 299-309 (1919).
12. Lincoln, S., Daly, M and Lander, E. Constructing genetic maps with Mapmakerexp 3.0. *Whitehead Institute Technical Report*. 3rd edition (1992).
13. Mace, E.S., Buhariwalla, H.K and Crouch, J.H.. A high-throughput DNA extraction protocol for tropical molecular breeding programs. *Plant Molecular Biology*. **21**: 459-460 (2003).
14. Murray, M and Thompson, W.F.. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*. **8**: 4321 - 4325 (1984).
15. Qi, X., Lindup, S., Pittaway, T.S., Allouis, S., Gale, M.D and Devos, K.M., Development of simple sequence repeat markers from bacterial artificial chromosomes without sub cloning. *Biotechniques*. **31**: 355-361 (2001).
16. Qi, X., Pittaway, T.S., Lindup, S., Liu, H., Waterman, E., Padi, F.K., Hash, C.T., Zhu, J., Gale, M.D and Devos, K.M.. An integrated genetic map of pearl millet, *Pennisetum glaucum*. *Theory of Application Genetics*. **109**: 1485-1493 (2004).
17. Quarrie, S.A., Steed, A., Calestani, C., Semikhodskii, A., Lebreton, C., Chinoy, C., Steele, N., Pljevljakusi, T.D., Waterman, E., Weyen, E.J., Schondelmaier, J., Habash, D.Z., Farmer, P., Saker, L., Clarkson, D.T., Abugalieva, A., Yessimbekova, M., Turuspekov, Y., Abugalieva, S., Tuberosa, R., Sanguineti, M.C., Hollington, P.A., Aragués, R., Royo, A and Dodig, D. A high-density genetic map of hexaploid wheat (*Triticum aestivum* L.) from the cross Chinese Spring x SQ1 and its use to compare QTLs for grain yield across a range of environments. *Theory of Application Genetics*. **110**: 865-880 (2005).
18. Rajaram, V., Nepolean, T., Senthilvel, S., Varshney, R.K., Vadez, V., Srivastava, R.K., Shah, T.M., Supriya, A., Kumar, S., Kumari, B.R., Bhanuprakash, A., Narasu, M.L., Lizarazu, O.R and Hash, C.T.. Pearl millet consensus linkage map constructed using four RIL mapping populations and newly developed EST-SSRs. *BMC Genomics*. **14**:159 (2013).
19. Senthilvel, S., Jayashree, B., Mahalakshmi, V., Kumar, P.S., Nakka, S., Nepolean, T and Hash, C.T., Development and mapping of simple sequence repeat markers for pearl millet from data mining of Expressed Sequence Tags. *BMC Plant Biology*. **8**: 119 (2008).
20. Sourdille, P. Transferable bread wheat EST-SSRs can be useful for phylogenetic studies among the *Triticeae* species. *Theory of Application Genetics*. **113**: 407-418 (2006).

21. Stam, P. Construction of integrated genetic linkage maps by means of computer package-JoinMap. *The Plant Journal.* **5:** 739-744 (1993).
22. Tai, T.H and Tanksley, S.D., A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Molecular Biology Reporter.* **8:** 297-303 (1990).
23. Thakur, R.P., Rai, K.N., Khairwal, I.S., Mahala, R.S. Strategy for downy mildew resistance breeding in pearl millet in India. *SAT ejournal.* **6:** 1-11 (2008).
24. Thakur, R.P., Shetty, K.G and King, S.B., Selection for host-specific virulence in asexual population of *Sclerospora graminicola*. *Plant Pathology.* **41:** 626-632 (1992)
25. Voorrips, R.E. Map Chart: software for the graphical presentation of linkage maps and QTLs.
26. *Journal of Heredity.* **93:** 77-79 (2002).
27. Young, N.D. A cautiously optimistic vision for marker-assisted breeding. *Molecular Breeding.* **5:** 505-510 (1999).